Werner protein recruits DNA polymerase δ to the nucleolus

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Werner syndrome is a Mendelian disorder of man that produces a number of manifestations resembling human aging. This disorder is caused by inactivation of the *wrn* gene, a member of the RecQ family of DNA helicases. The helicase and exonuclease activities of the Werner protein (WRN) suggest that it functions in DNA transactions, but the physiological function of WRN remains elusive. We present several lines of evidence that WRN interacts specifically with the p50 subunit of polymerase δ, the major DNA polymerase required for chromosomal DNA replication. P50, identified by yeast two-hybrid screening, interacts physically with the C terminus of WRN. Native WRN protein coimmunoprecipitates with p50 in a cellular fraction enriched in nucleolar proteins, and this immunocomplex also includes p125, the catalytic subunit of polymerase δ. In subcellular localization studies of cells transfected with WRN, p50 and p125 redistribute to the nucleolus and colocalize with WRN. These results suggest that one of the functions of WRN protein is to directly modify DNA replication via its interaction with p50 and abet dynamic relocalization of the DNA polymerase δ complexes within the nucleus.

Werner syndrome | DNA polymerase δ | nucleolus | yeast two-hybrid system

Werner syndrome (WS) is the single Mendelian disorder that most closely produces a phenotype resembling premature aging (1). The disorder is caused by mutations of the *wrn* gene, whose encoded protein is a member of the RecQ family of DNA helicases (2). Most *wrn* mutations produce C-terminal truncations resulting in impaired nuclear localization of the protein and functionally null alleles (3–5). Five human RecQ helicases are known, and three of them (WRN, BLM, and RecQL4) are implicated in single gene disorders, specifically WS, Bloom syndrome, and Rothmund–Thompson syndrome (6). These disorders display diverse clinical phenotypes; however, the molecular basis of these differences and precise *in vivo* function of these genes are largely unknown. Insight into the roles of WRN is of importance because it could reveal points of vulnerability underlying the stereotyped pattern of normal aging.

Recombinant WRN protein possesses ATP-dependent 3′-5′ DNA helicase activity (7). WRN unwinds specific quadruplexes in DNA (8) and can suppress the hyper-recombination phenotype exhibited by yeast mutants deficient in Sgs1, a homologue of RecQ-like genes (9). Although several RecQ family members exhibit similar biochemical features, WRN, uniquely to date, also has 3′-5′ DNA exonuclease activity (10, 11). Based on these enzymatic functions, WRN has been suggested to manipulate complex DNA structures arising during DNA replication and/or recombination.

Defects of DNA replication have been described in yeast *sgs1* mutants and also in WS. Earlier studies suggested that the S phase in WS cells was prolonged with reduced frequency of replication initiation (12, 13). Delayed replication fork progression also occurs in cells from Bloom syndrome patients (14, 15). Recent studies with *Xenopus* extracts have implicated FFA-1, the frog homologue of WRN in DNA replication focus formation (16). Despite the lack of phenotype in homozygous *wrn*-deficient mice, *wrn* −/− embryonic stem cells are sensitive to topoisomerase inhibitors (17). In addition, WRN associates with multiprotein DNA replication complexes prepared from wild-type mouse embryonic stem cells (17, 18).

Using the yeast two-hybrid screen, we found that the p50 subunit of the human DNA polymerase δ (Pol δ) interacts specifically with the C terminus of WRN *in vitro* and *in vivo.* Native WRN coimmunoprecipitates with p50 from the nucleolar fraction of human cells, and p125, the catalytic subunit of Pol δ, is also present in this immunocomplex. Furthermore, in subcellular localization studies, ectopic expression of WRN mobilizes both p50 and p125 to the nucleolus, and this effect is mediated by the C terminus of Werner protein. These studies suggest that WRN is directly coupled to replication fork progression and may be involved in regulating the initiation and progression of DNA replication by recruiting DNA polymerase δ to particular sites of DNA synthesis.

Materials and Methods

Plasmid Constructs. The bait containing the C terminus of WRN (WRNCT) encoding WRN amino acids 949-1401 was constructed by PCR amplifying a 1450-bp fragment from the full-length human *wrn* cDNA and cloned into a pAS2–1 yeast expression vector (CLONTECH) in frame with the GAL4 DNA binding domain. The pEGFP-WRN harboring the full-length coding region of the *wrn* gene was constructed as described (19). pEGFP-WRNCT (nt 3074–4530) was subcloned to pEGFP-C vector (CLONTECH). The green fluorescent protein (GFP)-tagged mouse Arf and human Bloom cDNAs were kindly provided by C. J. Sherr (St. Jude Hospital, Memphis, TN) and I. D. Hickson (Imperial Cancer Research Fund Laboratories, Oxford University, Oxford, U.K.), respectively.

Cell Lines and Culture. Mammalian cells were maintained in DMEM in the presence of 10% FBS, 2 mM glutamine, and 100 units/ml penicillin and streptomycin (GIBCO-BRL). The SV40-transformed control fibroblast cell line (WI-38/AG07217A) and Werner fibroblast cell line (AG11395/WS780, homozygous mutation of c.1336C→T) were obtained from the Coriell Institute for Medical Research.

Yeast Two-Hybrid Screening. The WRNCT bait was cotransformed intoY190 yeast cells with a human placental cDNA library fused to the GAL4 activation domain (CLONTECH HL4025AH).

Abbreviations: WS, Werner syndrome; WRN, protein encoded by the WS gene; BLM, protein encoded by the Bloom syndrome gene; Pol δ, DNA polymerase δ; WRNCT, C terminus of WRN; GFP, green fluorescent protein; PCNA, proliferating cell nuclear antigen; HA, hemagglutinin.

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The two-hybrid analysis was performed according to the manufacturer's recommendations. Five million independent clones of the placenta library were screened, and the cotransformants were selected on SD/Leu$^-$/Trp$^-$/His$^-$ plates in the presence of 25 mM 3-amino-1,2,4-triazole (3-AT, Sigma) followed by colony-lift assays for β-galactosidase activity. The LacZ+ phenotypes were scored by grouping the blue color intensities in comparison with the positive control after incubating the filters for maximum 8 h at 30°C. About 200 positive clones were obtained and subjected to a sequential selection process to eliminate false positive clones, according to the protocol by CLONTECH.

***In Vitro* Translation/Coimmunoprecipitation.** WRNCT fragment (nt 3074–4530) and full-length p50, the latter originating from a cDNA insert in the pACT2 yeast vector, were recloned into pcDNA3.1-His/Xpress and pcDNA 3.1-His/Myc expression vectors (Invitrogen), respectively. These constructs were transcribed and translated *in vitro* using the TNT T7-Coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S]methionine. The products were resolved by SDS/PAGE and fluorographed at −70°C after treatment with an enhancer (Enlightning, DuPont). The *in vitro* translated products were also verified by immunoblotting with the respective C terminus tag-specific antibodies and, for the full-length p50, with anti-p50 polyclonal antibody (gift of A.G. So, University of Miami). The coimmunoprecipitation studies were performed according to MacNeill *et al.* (20). [^{35}S]Methionine-labeled WRNCT polypeptide was incubated with either control or p50-Myc programmed nonradioactive reticulocyte lysates in the presence of anti-Myc or, as a control, anti-hemagglutinin (HA) polyclonal antibodies (both form Santa Cruz Biotechnology), and the immunocomplexes were captured by Protein-A-Sepharose (Amersham Pharmacia). To analyze the interaction of [^{35}S]methionine-labeled p50 with nonradioactive WRNCT, anti-Xpress (Invitrogen) and anti-HA (Boehringer) monoclonal antibodies and Protein G beads were used. The associated polypeptides and the input samples were resolved and visualized as above.

Immunoprecipitation and Immunoblotting. Extracts enriched in nuclear and nucleolar proteins were prepared as described (21) with minor modifications. HeLa cells, SV40-transformed control, and WS fibroblasts were harvested on ice with lysis buffer [10 mM Tris·HCl, pH 7.4/10 mM KCl/2 mM $MgCl_2$/0.05% Triton X-100/1 mM EGTA and protease inhibitors (Complete, Boehringer Mannheim)]. The nuclei were sonicated by 20 brief pulses on ice (output control 5, 20% duty cycle using Branson Sonifier 350) followed by centrifugation at 5,000 × *g* for 5 min. The supernatant (nucleoplasmic extract) was saved, and the nucleoli-enriched pellet was extracted in lysis buffer containing 300 mM KCl. After centrifugation, the supernatant (defined as nucleolar extract) was combined with the nucleoplasmic fraction, and Nonidet P-40 was added to a final concentration of 1%, or the nucleoplasmic and nucleolar extracts were processed separately with the ionic concentration of the respective lysates kept identical to those of the combined fraction. Protein G-Sepharose beads were preincubated with anti-WRN mouse monoclonal antibody developed against the extreme C terminus of the human WRN (Transduction Laboratories, Lexington, KY) or normal mouse IgG (Santa Cruz Biotechnology) in IP buffer (20 mM Tris·HCl, pH 7.4/150 mM NaCl/0.5 mM EDTA/1% Nonidet P-40 and protease inhibitors). In separate experiments, affinity-purified goat polyclonal antibody against the N terminus WRN (Santa Cruz Biotechnology) was used, or the extracts were incubated with protease-free DNase (1.5 units/ml) and RNase A (1.5 μg/ml) before immunoprecipitation. The antibody-coated beads were incubated with the extracts overnight at 4°C, then washed five times with IP buffer and boiled in 2× Laemmli sample buffer. Unprocessed cell extracts (pre-IP input) and immunocomplexes were subjected to Western blotting using polyvinylidene fluoride membranes (Immobilon-P, Millipore). The blots were probed with anti-p50 and anti-p125 polyclonal antibodies (latter kindly provided by P. Modrich, Duke University, and U. Hubscher, University of Zurich-Irchel) or anti-proliferating cell nuclear antigen (PCNA, Oncogene Research) and anti-WRN (Transduction Laboratories) monoclonal antibodies, followed by chemiluminescence detection.

Transfection and Immunofluorescent Microscopy. HeLa cells or SV40-transformed fibroblasts grown on coverslips were transiently transfected with the various plasmid DNAs using FuGene6 (Boehringer Mannheim). Sixty hours after transfection, the cells were fixed in PBS-buffered 4% formaldehyde/0.2% Triton X-100 at room temperature for 10 min. The coverslips were incubated with the primary antibodies in blocking solution (0.2% BSA in PBS) for 1 h at room temperature or overnight at +4°C. The following primary antibodies were used: anti-nucleolin monoclonal antibody (Medical & Biological Laboratories, Nagoya, Japan, 1:100), anti-Myc rabbit polyclonal antibody (Santa Cruz Biotechnology, 1:100), affinity-purified rabbit polyclonal antibody against human p50 (0.04 mg/ml), affinity-purified rabbit polyclonal antibody against p125 (0.05 mg/ml, gift of U. Hubscher), and affinity-purified rabbit polyclonal antibody against the C terminus of human WRN (1:30, ref. 22). After washing, the cells were incubated with fluorescent isothiocyanate (FITC), Texas Red, or rhodamine-conjugated secondary antibodies (The Jackson Laboratory). DNA was visualized with 4,6-diamino-2-phenylindole included in the anti-fade mounting medium (Vector Laboratories). Immunofluorescence was detected using an Axiovert 135 (Zeiss) fluorescent microscope. Bio-Rad MRC 600 or Zeiss LSM510 confocal laser scanning microscopes were used for simultaneous collection of signals for direct GFP expression and for immunofluorescence of single 0.25 μm optical sections.

Results

Identification of p50, the Second ("Small") Subunit of DNA Polymerase δ, as a Protein-Interacting Partner of WRN *in Vitro*. The yeast two-hybrid system was used to screen a human placental cDNA expression library with a C-terminal 450-amino acid fragment of the WRN protein (WRNCT). This bait encompasses almost the entire C terminus, including the nuclear localization signal but not the conserved RecQ domain (Fig. 1*A*). Twenty-two independent clones showing specific interaction with the WRNCT bait were isolated. One of these encoded the full-length p50 protein, the second ("small") subunit of DNA polymerase δ, which is itself devoid of enzymatic activity but required for the maintenance of highly processive DNA synthesis by Pol δ (reviewed in ref. 23). The interaction of p50 with WRNCT was specific, as cotransformation of the p50 cDNA with the yeast expression vector harboring the GAL4-DNA binding domain alone or with cDNAs encoding unrelated proteins was inactive (Fig. 1*B*).

To confirm the specific interaction between the C terminus WRN and p50 by an independent method, we subcloned the corresponding cDNAs into expression vectors that permit *in vitro* transcription/translation and provide C terminus tags of X-Press and Myc for WRNCT and p50, respectively. The [^{35}S]methionine-labeled *in vitro* translated WRNCT was incubated with nonradioactive p50-Myc fusion protein followed by immunoprecipitation using anti-Myc or the unrelated anti-HA antibodies. The ^{35}S-labeled WRNCT was coprecipitated by anti-Myc (Fig. 1*C*, lanes 1 and 2) but not by anti-HA (lanes 3 and 4). Reverse immunoprecipitation experiment using ^{35}S-labeled p50 and nonradioactive WRNCT polypeptides confirmed this finding (data not shown). These results indicate that the C-terminal portion of WRN and p50 undergoes a specific, direct interaction *in vitro*.

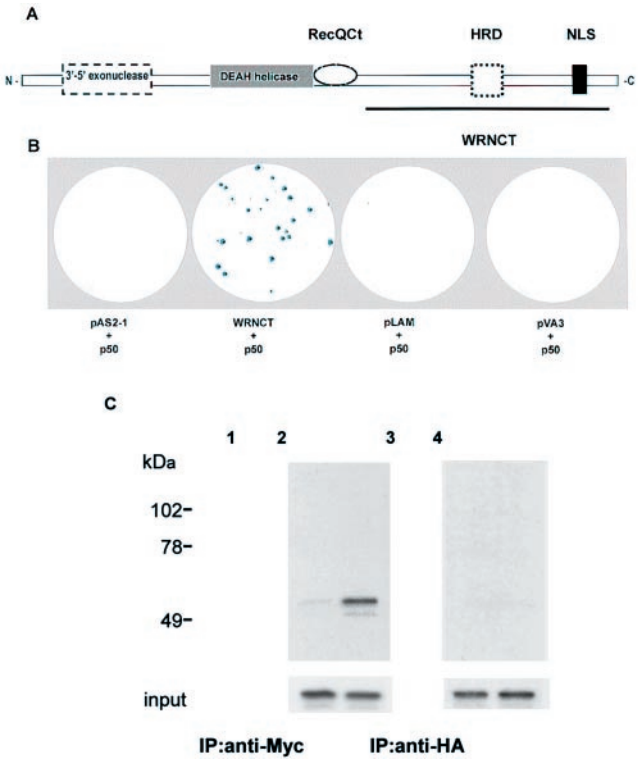

Fig. 1. p50 protein associates with the C terminus of WRN *in vitro*. (*A*) Yeast two-hybrid screening. The WRNCT bait encoding amino acids 949-1401 (~50 kDa) of the human WRN protein was cloned into pAS2–1 vector and used to screen a human placental cDNA library. The schematic representation of the WRNCT bait shows the C terminus nuclear localization signal (NLS), the conserved RecQ domain (RecQCt), and the putative nucleic acid binding domain (HRD). (*B*) Retransformation of the yeast expression plasmids to Y190 host strain. pAS2–1, bait vector alone; p50, full-length p50 in pACT2 vector in fusion with GAL4-activator domain. pLAM and pVA3 are human lamin C and murine p53, respectively, cloned into the pAS2–1 vector. (*C*) *In vitro* translation combined with immunoprecipitation. WRNCT and full-length p50 cloned into pcDNA3.1-His/Xpress and pcDNA 3.1-His/Myc vectors, respectively, were transcribed/translated *in vitro* in the presence (WRNCT) or absence (p50) of [^{35}S]methionine. Equal amounts of labeled WRNCT polypeptide were mixed with either control (lanes 1 and 3) or p50-Myc programmed nonradioactive reticulocyte lysates (lanes 2 and 4) and immunoprecipitated by anti-Myc (lanes 1 and 2) or anti-HA (lanes 3 and 4) polyclonal antibodies. The associated polypeptides were resolved by SDS-PAGE and visualized by autoradiography. The lower panel indicates 2.5% of the corresponding mixtures loaded before immunoprecipitation (input). We estimated that 4–5% of the input protein could be coprecipitated with the anti-Myc antibody used.

Native WRN and p50 Associate *in Vivo*. To obtain evidence that endogenous WRN and p50 form a complex *in vivo*, a modified subcellular fractionation method (21) resulting in highly enriched nuclear and nucleolar proteins was used to prepare extracts from different cell lines. These extracts were immunoprecipitated with a monoclonal antibody against the extreme C terminus of WRN, followed by immunoblotting with anti-p50 polyclonal antibody. Both in HeLa cells and SV40-transformed WI-38 control fibroblasts, the endogenous p50 was detected in the WRN immunoprecipitate (Fig. 2 *Upper*, lanes 2, 4, and 6) but not when normal IgG (lanes 1 and 3) or anti-HA monoclonal antibody (data not shown) was used as controls. No detectable p50 was coprecipitated by anti-WRN in extracts of SV40-transformed fibroblasts from a WS patient with undetectable endogenous WRN (Fig. 2 *Upper*, lanes 5 and 6; ref. 5). The abundance of endogenous p50 was similar in both control and WRN-deficient cells (Fig. 2. *Lower*, input lanes). Immunoprecipitation using a polyclonal antibody directed against the N terminus of the human WRN protein gave identical results.

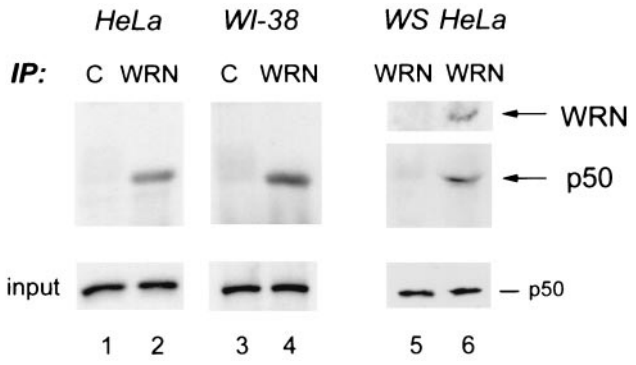

Fig. 2. Coimmunoprecipitation of endogenous WRN with p50. Extracts enriched in nuclear and nucleolar proteins were prepared from HeLa cells, SV40-transformed normal human fibroblasts (WI-38), or fibroblast derived from Werner syndrome patients (WS). Aliquots containing approximately 800 μg protein were immunoprecipitated (IP) with a monoclonal antibody against the human WRN protein (WRN) or with normal mouse IgG as control (*C*). Western blot analysis was performed using anti-p50 (*Upper*, lanes 1–6) and anti-WRN antibodies (lanes 5 and 6, indicated separately). The lower panel indicates immunoblotting of the respective unprocessed extracts using anti-p50 antibody (input, lanes 1–6).

The association of WRN and p50 was still present after treatment of the extracts with DNase I and RNase A, suggesting that this protein–protein interaction does not depend on binding of the protein complex to nucleic acids (data not shown).

WRN Recruits p50 to the Nucleolus. The coimmunoprecipitation of WRN and p50 from human cell extracts indicates these proteins forming a complex *in vivo*. Based on *in vitro* experiments (Fig. 1), the C terminus of WRN seems to be sufficient for association with p50. To understand the significance of this protein–protein interaction at the cellular level, we transiently transfected plasmids encoding GFP-tagged forms of WRNCT, full-length WRN, and the Myc-tagged full-length p50 into asynchronous HeLa cells and analyzed their subcellular distribution. Immunoblotting with anti-GFP and anti-Myc antibodies confirmed that after transfection both WRNCT and p50 were expressed at their predicted sizes (data not shown). Transfection of the full-length WRN construct used in the current experiments produces a stable fusion protein and functionally complements the increased sensitivity of WS fibroblasts to the genotoxin 4NQO (19).

GFP alone distributed diffusely in the nucleus and cytoplasm (Fig. 3*Aa–c*), whereas GFP-tagged WRNCT localized to the nucleus with predominant accumulation in the nucleolus (Fig. 3*Ad*) and colocalized with the major nucleolar protein, nucleolin (Fig. 3*Ad–f*). The full-length WRN showed a similar subcellular distribution (Fig. 3*Ag–i*), indicating that the nucleolar localization of WRN is mediated, at least in part, by its C terminus. The observed nucleolar localization of WRN is consistent with the distribution of endogenous WRN protein in several cell types (22, 24, 25). Overexpressed p50, detected by the tag-specific anti-Myc antibody, was localized in the nucleoplasm excluding the nucleolus in over 95% of the transfected cells (Fig. 3*Aj–l*). By contrast, coexpression of GFP-WRNCT invariably recruited the ectopically expressed p50 into the nucleolus, where it colocalized with WRNCT (Fig. 3*Bd–f*). Ectopically expressed full-length WRN also mobilized p50 to the nucleoli (Fig. 3*Bg–i*). Similar results were obtained using COS-7 cells and SV40-transformed normal WI-38 fibroblasts (data not shown).

Nucleolar recruitment of proteins has recently been recognized as a regulatory mechanism involved in key cellular processes (reviewed in ref. 26). Both mouse and human ARF tumor suppressor proteins are known to mobilize and sequester the

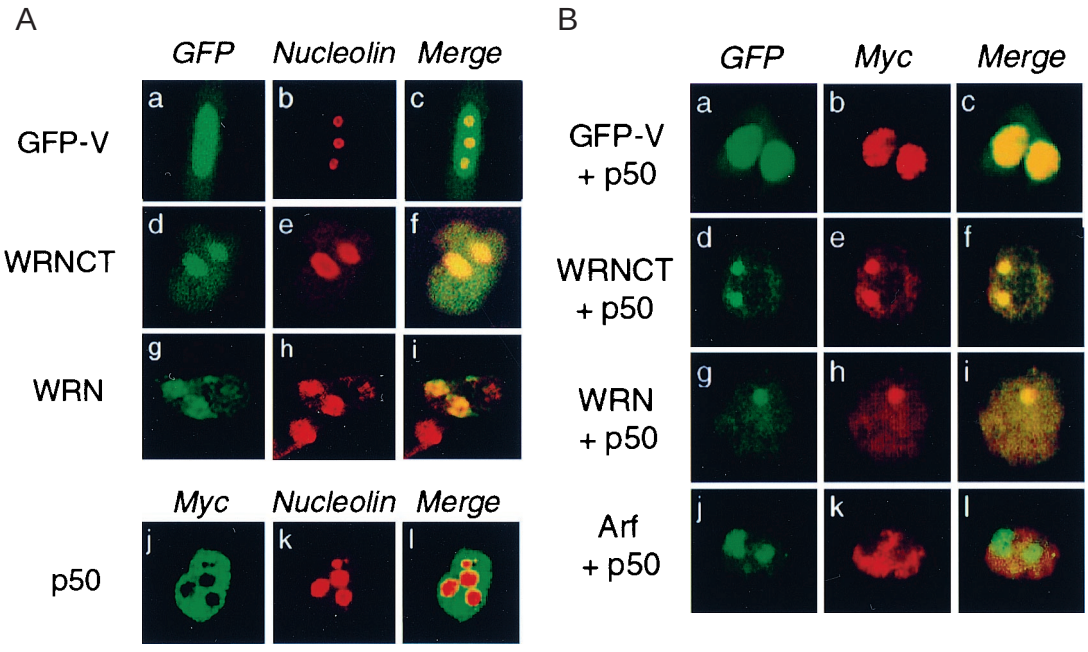

Fig. 3. Ectopic expression of WRN mobilizes p50 from the nucleoplasm to the nucleolus. (*A*) Exponentially growing asynchronous HeLa cells were transiently transfected with pEGFP vector (*a–c*), WRNCT (*d–f*), and full-length WRN cloned in frame to the C terminus of EGFP (*g–i*) or Myc-tagged full-length p50 (*j–l*). The cells were imaged by confocal fluorescent microscopy directly visualizing GFP expression (green, *a*, *d*, and *g*) or using indirect immunofluorescence with mouse monoclonal antibody against nucleolin (red, *b*, *e*, *h*, and *k*) or rabbit polyclonal antibody against the Myc epitope (green, *j*) followed by Texas Red or FITC-conjugated secondary antibodies, respectively. Merged images show that WRNCT and full-length WRN localize predominantly to the nucleolus (overlapping regions yellow, *f* and *i*), whereas p50 localizes to the nucleoplasm excluding the nucleolus (*l*). (*B*) Transiently coexpressed WRNCT and p50 colocalize in the nucleolus (*d–f*). Similar pattern is seen when GFP-tagged full-length WRN is coexpressed with p50-Myc (*g–i*). GFP-tagged mArf localizes to the nucleolus (green, *j*) but fails to relocalize p50 (*k* and *l*).

negative regulator MDM2/HDM2 to the nucleolus providing a mechanism for p53 activation (27, 28). Also, cross-talk between WRN and p53 has been proposed (29, 30). To demonstrate the specificity of WRN in recruiting p50 to the nucleolus, we examined whether exogenous expression of $p19^{Arf}$ alters the subnuclear localization of p50. Full-length GFP-tagged mouse $p19^{Arf}$ cDNA was transiently transfected alone or simultaneously with Myc-tagged p50 into HeLa cells. GFP-mArf compartmentalized in the nucleoli (Fig. 3*Bj*) but failed to mobilize exogenous p50 to the nucleolus (Fig. 3 *Bk* and *Bl*) or perturb the nucleolar accumulation of endogenous WRN protein (data not shown). These findings suggest that WRN protein, via its C terminus, specifically interacts with p50 *in vivo* in human cells and can relocalize this Pol δ subunit within the nucleus, thus defining an intracellular molecular effect for WRN.

WRN Associates with Endogenous Components of the Core Pol δ Enzyme in the Nucleolus. To determine whether WRN is able to mobilize endogenous p50 to the nucleolus in the absence of p50 overexpression, HeLa cells were transfected with GFP-WRNCT, and the subnuclear distribution of endogenous p50 was examined. Native p50 was excluded from the nucleoli in the significant majority (over 90%) of nuclei visualized (Fig. 4, g′). As in the case of ectopic p50 (Fig. 3*Bd–f*), WRNCT recruited endogenous p50 to the nucleolus (Fig. 4*a–c*). On the other hand, the distribution pattern of endogenous p50 was not changed upon the overexpression of GFP-tagged full-length BLM, the closely related RecQ-like DNA helicase mutated in Bloom syndrome (Fig. 4*d–f*). The observed nuclear signal of GFP-BLM, disperse punctate foci in greater than 90% of transfected cells with additional rare, larger nuclear patches (<5%), was comparable with the previously described distribution of endogenous BLM (25, 31). Together, these findings indicate that relocalization of p50 by WRN occurs under physiological circumstances, and this interaction seems to be specific for WRN and not due to domains conserved between the two related helicases, BLM and WRN.

p50 and the catalytic subunit p125 constitute the dimeric, active core Pol δ in mammalian systems responsible for most DNA synthesis (reviewed in refs. 23 and 32). Next, we examined whether the subnuclear distribution of p125 changes upon p50 mobilization to the nucleolus. Endogenous p125 displayed a diffuse nucleoplasmic distribution with apparent exclusion of nucleoli (Fig. 4, h′), and it colocalized with overexpressed p50 (Fig. 4*g–i*). Exogenously expressed WRNCT redistributed endogenous p125 to the nucleolus in about 30–40% of the cells (data not shown). Cotransfection of WRNCT and full-length p50, however, invariably produced an accumulation of the endogenous p125 immunoreactivity in the nucleolus with residual nucleoplasmic immunoreactivity evident (Fig. 4*j–l*).

We inquired whether additional components of Pol δ and the DNA replication complex are present in WRN immunoprecipitates and whether the WRN complex preferentially exists in distinct subcompartments within the nucleus, as suggested by colocalization of WRN with p50 and p125 in the nucleolus (Figs. 3 and 4). Separate nucleoplasmic and nucleolar extracts were prepared from HeLa cells and immunoprecipitated with anti-WRN monoclonal antibody. Fig. 5*A* demonstrates that WRN coprecipitated native p50 in the nucleolar fraction (*Middle*, lane 4). p125, the catalytic subunit of Pol δ, was present in the same immunocomplex (*Top*). The p50 signal detected in the pre-IP nucleolar extract appears as a doublet (Fig. 5*A Bottom*, lanes 2 and 4). The two bands may represent different posttranslationally modified forms of the protein or distinct p50 isoforms. As shown recently (18), proliferating cell nuclear antigen (PCNA), the major accessory protein required for highly processive Pol δ activity, also coimmunoprecipitated with WRN using nuclear

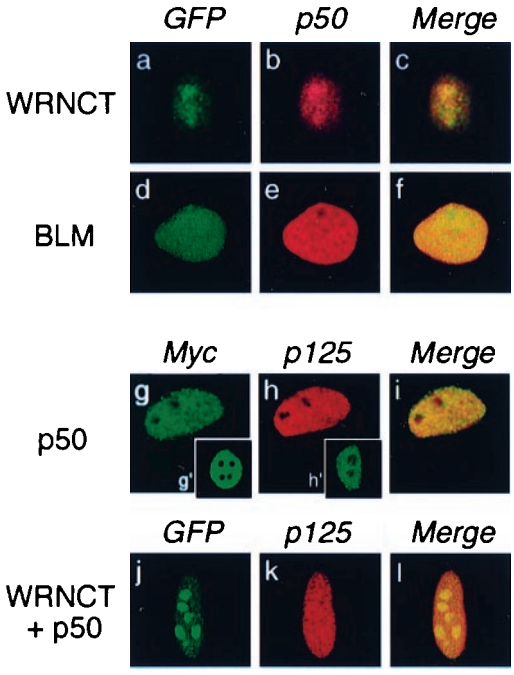

Fig. 4. The components of the endogenous Pol δ core enzyme are recruited to the nucleolus by WRN. HeLa cells were transiently transfected with GFP-tagged cDNAs encoding WRNCT or full-length BLM (green, *a* and *d*) and stained with an affinity-purified polyclonal antibody against p50. Exogenously expressed WRNCT, but not BLM, recruits the endogenous p50 to the nucleolus (red, *b* and *e*, merged images *c* and *f*, respectively). Double immunostaining with anti-Myc and anti-p125 shows that overexpressed p50 (green, *g*) colocalizes with the endogenous p125 (red, *h*, merged image yellow, *i*). Endogenous p50 and p125 staining in untransfected cells are shown in insets (green, g′ and h′). Cotransfection of WRNCT and p50 leads to partial relocalization of p125 to the nucleolus (*j–l*).

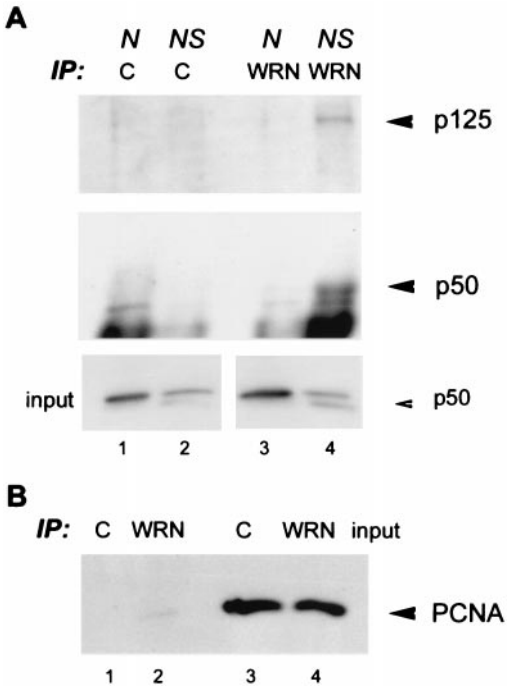

Fig. 5. Association of WRN with components of the core Pol δ enzyme *in vivo*. (*A*) Separate nucleoplasmic (N) and nucleolar (NS) fractions were prepared and immunoprecipitated with normal mouse IgG as control (C) or anti-WRN (WRN) monoclonal antibody. The precipitates were analyzed by Western blotting using anti-p50 antibody (*Middle*, lanes 1–4). The same blot was reprobed by anti-p125 polyclonal antibody (*Top*). The bottom panel indicates the respective unprocessed extracts probed with anti-p50 (input, lanes 1–4). (*B*) Combined nuclear and nucleolar extracts from HeLa cells were immunoprecipitated with normal mouse IgG (C) or anti-WRN monoclonal antibody (WRN) and analyzed by Western blotting with a monoclonal antibody against human PCNA (lanes 1 and 2). Pre-IP inputs for the respective immunoprecipitates are indicated (lanes 3 and 4).

extracts enriched in nucleolar proteins (Fig. 5*B*). Compared with their pre-IP input signals, WRN precipitates seem to contain relatively large amounts of p50, lesser amounts of the p125, and very small amounts of PCNA (Figs. 2 and 5 *A* and *B*), suggesting that association of PCNA with WRN may be partly indirect.

Together, these data strongly suggest that redistribution of p125, the catalytic subunit of Pol δ, to the nucleolus is a sequel of the interaction between WRN and p50, and both components of the native core Pol δ associate in a complex with WRN.

Discussion

We have shown that p50, an essential subunit of the human DNA polymerase δ involved principally in DNA replication, forms a direct physical association with the C terminus of WRN *in vitro* and interacts with WRN protein *in vivo*. Subcellular localization studies reveal that WRN can redistribute nuclear p50 and p125, the catalytic subunit of Pol δ, to the nucleolus. This effect is mediated, at least in part, by the C terminus of WRN protein. Moreover, the *in vivo* interaction of native proteins is demonstrated by coimmunoprecipitation studies, where native WRN seemed to form a complex with p50 and p125 predominantly in a subcellular fraction highly enriched in nucleolar proteins. These experiments provide a molecular link to the findings that WRN may be part of the DNA replication machinery (16–18) and that in WS cells the S phase is prolonged due to defective replication initiation and elongation (13). Furthermore, these studies suggest that p50, whose function is largely undefined, may play a role in targeting proteins to the polymerase δ complex and targeting the complex within the nucleus.

The mobilization of the core components of Pol δ to the nucleolus suggests that dynamic protein recruitment to distinct subcompartments within the nucleus may be an integral part of the function of WRN. This may represent another example of a recently emerging theme: spatial and temporal relocalization of proteins within the nucleus serves as a regulatory mechanism governing key processes, such as cell-cycle progression or tumorigenesis (reviewed in ref. 26). Endogenous WRN protein localizes predominantly in the nucleolus in human primary fibroblasts or transformed human cell lines without apparent cell-cycle dependence (22, 24, 25). However, the role of this subcellular localization is unknown. Our results raise the possibility that WRN influences a dynamic spatial redistribution of the Pol δ within the nucleus under physiological conditions. Basic amino acid stretches suggestive of nucleolar targeting signals (33) are present within the C terminus of WRN, and these are, in part, distinct from the described nuclear localization signal (4). Whether regions or critical residues of p50, other than those involved in the interaction with WRN, are also required to the mobilization of this Pol δ subunit into the nucleolus remains to be determined.

Native WRN and p50 can colocalize in the nucleolus in a small subset of asynchronized, untransfected HeLa or fibroblast cell lines (unpublished observation). The precise cellular conditions leading to the nucleolar accumulation of endogenous p50 is a subject of current studies. Importantly, using subnuclear fractionation and immunoprecipitation, a large proportion (approximately 50%) of native p50 in the nucleolar fraction was associated with WRN (Fig. 5*A*). Whereas electron microscopic studies of this fraction confirm

that it is highly enriched in nucleoli (21), the biochemically defined nucleolar fraction may also include extranucleolar chromatin components containing p50:WRN complexes. Further definition of these components would be of interest.

Recent *in vitro* biochemical data indicate that both human and *Schizosaccharomyces pombe* Pol δ consist of at least four subunits forming a complex of dimers of heterotetramers (34, 35). Although in reconstituted systems the core Pol δ enzyme, comprising yeast homologues of p125 and p50, may be less efficient than the holoenzyme, p50 is essential for yeast viability and for the stimulatory effect of PCNA on processive DNA synthesis both in yeast and mammalian system (20, 36, 37). While this manuscript was in preparation, a report appeared showing that human WRN stimulates DNA synthesis by a reconstituted Pol δ from *Saccharomyces cerevisiae in vitro* (38). These experiments provide functional evidence that WRN interacts with this major replicative polymerase and raise the possibility that additional Pol δ subunits may contribute to the effect of WRN on the activity of this enzyme complex. It can be of further importance that the exonuclease activity of WRN (10, 11), which is unique among RecQ helicases, may function in concert with DNA polymerase δ, perhaps in removing nucleotides during DNA replication.

Our experiments suggest that p50 may directly link WRN protein to the DNA replication machinery. Even subtle alterations of DNA replication in the absence of WRN protein may have significant consequences. In light of the emerging view that the pathways of DNA replication, recombination and repair are intimately related, WRN protein may function at several levels. It may regulate the recruitment of DNA polymerase δ to selected replication sites, thereby exerting a dynamic control of replication origin use. WRN may facilitate replication progression by resolving complex GC-rich secondary structures (8). As recently shown for certain RecQ helicases, WRN may also be involved in recombination-mediated replication restart at forks blocked either by DNA damage or abnormal secondary structures (reviewed in ref. 39). At this juncture, it is unknown whether WRN has any function on DNA transactions within the nucleolus itself. Perhaps WRN may sequester DNA polymerase δ complexes with distinct composition in the nucleolus (or associated chromatin), and these complexes may be then mobilized by precise cellular conditions to specific sites within the nucleus.

Recent work suggests that a functional interaction between WRN and telomerase may exist (19, 40). WRN protein can also interact with the Ku complex (41), a heterodimer involved in DNA repair and telomere maintenance. As Pol δ is also required for telomere elongation *in vivo* (42), the association of WRN with components of Pol δ may have implications for telomere replication. WRN may be involved in preparing telomere ends either by expediting the assembly/disassembly of T-loops, mobilizing Pol δ to the ends of chromosomes, and/or forming an end-replication complex, in analogy to replisomes that form at origins of replication and primosomes that form at DNA forks (reviewed in ref. 43). An extension of this hypothesis is that lack of WRN may hinder uniform telomere replication and/or T-loop assembly/disassembly, resulting in signals for cell-cycle arrest. Once a cell enters into the senescence, removal of the original stimulus for this may not be sufficient to reverse the phenotype. The challenge remains to define the precise molecular role of WRN and decipher the mechanisms whereby WRN participates in spatial and temporal organization of cellular processes that may underlie aging.

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